11-9-2009

Greater Apparent Absorption of Flavonoids Is Associated with Lesser Human Fecal Flavonoid Disappearance Rates

Andrean L. Simons
Iowa State University

Mathieu Renouf
Iowa State University

Patricia A. Murphy
Iowa State University, pmurphy@iastate.edu

Suzanne Hendrich
Iowa State University, shendric@iastate.edu

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 141–147 141
DOI:10.1021/jf902284u

Greater Apparent Absorption of Flavonoids Is Associated with Lesser Human Fecal Flavonoid Disappearance Rates

Andrean L. Simons, Mathieu Renouf, Patricia A. Murphy, and Suzanne Hendrich*

Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa 50011

It was hypothesized that 5,7,4′-OH-flavonoids disappeared more rapidly from human fecal incubations and were less absorbable by humans than flavonoids without 5-OH moieties. Anaerobic fecal disappearance rates over 24 h were determined for 15 flavonoids in samples from 20 men and 13 women. In these anaerobic fecal mixtures, flavonoids with 5,7,4′-OH groups, genistein, apigenin, naringenin, luteolin, kaempferol, and quercetin (disappearance rate, $k = 0.46 \pm 0.10$ h^{-1}), and methoxylated flavonoids, hesperetin and glycitein ($k = 0.24 \pm 0.21$ h^{-1}), disappeared rapidly compared with flavonoids lacking 5-OH (e.g., daidzein, $k = 0.07 \pm 0.03$ h^{-1}). Apparent absorption of flavonoids that disappeared rapidly from in vitro fecal incubations, genistein, naringenin, quercetin, and hesperetin, was compared with that of daidzein, a slowly disappearing flavonoid, in 5 men and 5 women. Subjects ingested 104 μmol of genistein and 62 μmol of daidzein (soy milk), 1549 μmol of naringenin and 26 μmol of hesperetin (grapefruit juice), and 381 μmol of quercetin (onions) in three test meals, each separated by 1 week. Blood and urine samples were collected over 24 h after each test meal. Plasma flavonoid concentrations ranged from 0.01 to 1 μM. The apparent absorption, expressed as percentage of ingested dose excreted in urine, was significantly less for naringenin (3.2 ± 1.7%), genistein (7.2 ± 4.6%), hesperetin (7.3 ± 3.2%), and quercetin (5.6 ± 3.7%) compared with daidzein (43.4 ± 15.5%, $p = 0.02$). These data affirmed the hypothesis that the 5,7,4′-OH of flavonoids limited apparent absorption of these compounds in humans.

KEYWORDS: Flavonoid; isoflavone; bioavailability; metabolism; human

Flavonoids are polyphenolic compounds that are widely distributed in foods of plant origin (*1*). Daily intakes in humans range from a few milligrams to 1 g (*2*). Flavonoids in fruits and vegetables have been suggested to lower the risk of steroid-dependent cancers (*3*). A recent meta-analysis showed that cocoa, tea, and soy flavonoid intake reduced several heart disease risk factors (*4*) in populations consuming large amounts of flavonoids, but other flavonoids are not well-studied to date. Over 5000 flavonoids have been identified to date and are divided into subclasses, which differ in their heterocyclic C ring (*5*), including flavones, flavanones, flavonols, and isoflavones (**Figure 1**). Substitution patterns on the A and B rings with hydroxyl, methyl, methoxyl, O- and C-sugars, acyl, prenyl, sulfate, and glucuronide groups provide additional structural variation in each flavonoid subclass (*5*).

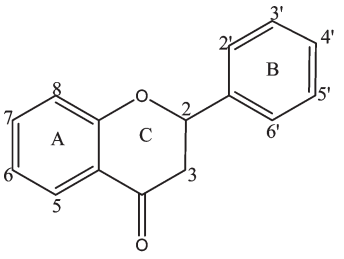
Flavonoid glycosides are their predominant forms in foods. The glycosides are absorbed to a very limited extent (*5*) and are cleaved by gut bacterial or human intestinal β-glycosidases (*6*). Flavonoid aglycons are absorbed across the intestinal mucosa and conjugated in the mucosa and liver by phase II enzymes (UDP-glucuronosyltransferase, sulfotransferase, and catechol-*O*-methyltransferase) (*7*). The flavonoids may be excreted in the urine or bile. Bacteria in the lower intestine hydrolyze the flavonoid conjugates after biliary excretion, which results in reabsorption of the flavonoid aglycons and enterohepatic recirculation (*7*,*8*). The gut bacteria apparently also further degrade the aglycons (*9*).

Determination of the metabolism and bioavailability of flavonoids is crucial in the assessment of their health effects, especially knowing the extent of absorption of intact flavonoid aglucons. Apparent absorption and plasma concentrations of isoflavones were strongly related to fecal contents of these compounds. Two women who excreted 10-fold more isoflavones in feces than did five other women also showed different apparent absorption of isoflavones; plasma daidzein and genistein were similar in the two high fecal isoflavone excreters, but daidzein was apparently absorbed to a significantly greater extent than genistein in the low fecal excreters (*10*). This laboratory has developed in vitro human fecal fermentation systems to determine flavonoid disappearance rate (*10*), identify fecal isoflavone metabolites, and predict human bioavailability of these compounds (*10*−*13*). Slow in vitro fecal disappearance rates of daidzein and genistein corresponded to greater daidzein and genistein bioavailability in human subjects, measured by the mean amount of isoflavones recovered in urine as a percentage of ingested dose (*11*). The rate

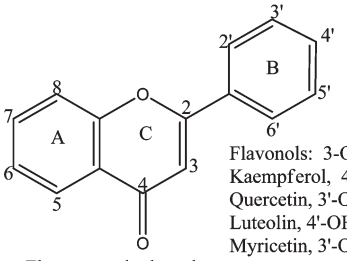
*Address correspondence to this author at 220 MacKay, Iowa State University, Ames, IA 50011-1123 [telephone (515) 294-4272; fax (515) 294-6193; e-mail shendric@iastate.edu].

Published on Web 11/18/2009

of fecal isoflavone disappearance in vitro depended on the chemical structure of isoflavones (*10*, *11*). Genistein, which has a 5-OH, was degraded significantly nore quickly than daidzein, which does not possess this structural feature (*10*), and in most subjects, daidzein was significantly more bioavailable than genistein, as reflected in urinary excretion (*10*, *13*).

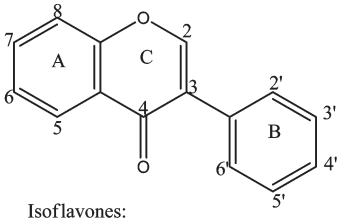


Flavanones:
Hesperetin, 3'-OH, 4'-OCH_3, 5-OH, 7-OH,
Naringenin, 4'-OH, 5-OH, 7-OH



Flavonols: 3-OH
Kaempferol, 4'-OH, 5-OH, 7-OH
Quercetin, 3'-OH, 4'-OH, 5-OH, 7-OH
Luteolin, 4'-OH, 5'-OH, 5-OH, 7-OH
Myricetin, 3'-OH, 4'-OH, 5'-OH, 5-OH, 7-OH

Flavone, no hydroxyls
Other flavones:
Chrysin, 5-OH, 7-OH
Apigenin, 4'-OH, 5-OH, 7-OH



Isoflavones:
Daidzein, 4'-OH, 7-OH
Glycitein, 4'-OH, 6-OCH_3,7-OH
Genistein, 4'-OH, 5-OH, 7-OH

Figure 1. Flavonoid structures and numbering system.

The chemical structure and substitution pattern of other flavonoids, flavones, flavanones, and flavonols influenced their in vitro anaerobic fecal disappearance rates (*14*). Although numerous gut microbial metabolites may be formed from flavonoids, most evidence of bioactivity to date has focused on the parent flavonoids. A recent study of flavonoids found predominantly in tea and onions showed that the plasma contents of the various gut microbial flavonoid metabolites were insignificant compared with the parent flavonoid aglycons and suggested that the main biological effects of the flavonoids are probably attributable to the parent compounds (*15*). In the present study, we hypothesized that the chemical structure of flavonoids determined their rate of fecal disappearance in vitro and their apparent absorption in humans.

MATERIALS AND METHODS

Subjects. Thirty-three subjects (20 male and 13 female) were recruited from Iowa State University for the in vitro flavonoid degradation study. The subjects' ages ranged from 18 to 37 years (mean age = 25.6 ± 4.4 years) with a body mass index (BMI) of 18.1−46.1 kg/m^2 (mean BMI = 23.7 ± 4.9 kg/m^2), respectively. The ethnicities of the subjects included 15 Caucasians, 7 Asian Indians, 7 Chinese, 3 African Americans, and 1 African.

Ten (5 male and 5 female) of the 33 subjects participated in the human bioavailability studies. These subjects were selected on the basis of a moderate or low in vitro fecal daidzein disappearance phenotype (average k = 0.053 ± 0.029 h^{-1}) to attempt to limit interindividual variability in flavonoid bioavailability (*13*). These subjects were 18−30 years of age (mean age = 25.0 ± 4.0 years) with BMI of 18.1−29.1 kg/m^2 (mean BMI = 22.6 ± 3.3). The ethnicities included three Caucasians, three Chinese, two Asian Indians, and two African Americans (**Table 1**). All subjects were healthy and not taking any medication. Approval of the study design was obtained from the Iowa State University Human Subjects Research Committee in 2004. The subjects followed an isoflavone-, flavonol-, and flavanone-free diet for 1 week before their respective feedings. All subjects were given oral and written instructions on foods and beverages not to consume during each washout period based on the flavonoid levels reported in the USDA database for the flavonoid content of selected foods (*16*).

Reagents and Chemicals. Apigenin, naringenin, kaempferol, luteolin, quercetin, flavone, chrysin, 7,4′-dihydroxyflavone, 6,4′-dihydroxyflavone, 5,4′-dihydroxyflavone, and 5,3′-dihydroxyflavone were from Indofine Chemical Co., Inc. (Hillsborough, NJ). Daidzein and 2,4,4′-trihydroxydeoxybenzoin (THB) were synthesized using the method of Song et al. (*17*). Genistein was synthesized according to a modification of the method of Chang et al. (*18*). HPLC grade acetonitrile,

Table 1. Subject Characteristics

subject ID	sex	BMI (kg/m^2)	age (years)	gut transit time (h)	in vitro anaerobic fecal daidzein degradation rate, k (h^{-1})	ethnicity
114	F	23.2	22	40 ± 9	0.054	Caucasian
118[a]	F	18.1	24	53[c]	0.120	Asian
119	F	22.7	30	67 ± 20	0.047	Asian
125	F	29.1	24	50[c]	0.037	African American
127[b]	F	22.5	26	104[c]	0.046	African American
206	M	26.3	24	39 ± 6	0.017	Caucasian
212	M	20.7	18	23 ± 16	0.036	Asian Indian
217	M	20.7	30	23 ± 11	0.064	Asian
224	M	23.7	25	75[c]	0.030	Caucasian
229	M	19.0	27	65 ± 9	0.074	Asian Indian
female		23.1 ± 3.9	25 ± 3	63 ± 25	0.061	
male		22.1 ± 2.9	25 ± 4	45 ± 24	0.044	
overall		22.6 ± 3.3	25 ± 4	54 ± 25	0.053 ± 0.029	

[a] Dropped out after the soy milk feeding period from discomfort due to blood withdrawal. [b] Dropped out during the onion feeding period. [c] Missing standard deviation values due to difficulty observing the red dye or incomplete collection of samples.

methanol, acetic acid, dimethyl sulfoxide (DMSO), and all other chemicals were from Fisher Scientific (Fair Lawn, NJ). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used to prepare all solutions.

Flavonoid Degradation and Bioavailability Study Designs. All microbial fermentation experiments to determine flavonoid degradation rates were performed according to the method of Simons et al. (*14*), except that the final concentration of each flavonoid was 100 μmol/L; incubations were performed anaerobically over 24 h. The human bioavailability studies of isoflavone, flavanone, and flavonol were performed on three separate days with a 2 week washout period between each feeding. To attempt to minimize intersubject variability, subjects were prescreened according to fecal daidzein degradation rates, which separated into three significantly different phenotypes by statistical cluster analysis: high (k = 0.40 ± 0.14 h^{-1}, n = 3), moderate (k = 0.10 ± 0.02 h^{-1}, n = 10) and low (k = 0.04 ± 0.02 h^{-1}, n = 20, $p < 0.05$). Ten of the 30 subjects showing moderate and low fecal daidzein degradation phenotypes were recruited to ingest flavonoid test meals (**Table 1**). The isoflavones genistein and daidzein were provided from soy milk, flavanones naringenin and hesperetin were from grapefruit juice, and the flavonol quercetin was from sautéed red onions. The ingested amount of soy milk or grapefruit juice was 2 cups, containing 28 mg of genistein and 16 mg of daidzein, or 422 mg of naringenin and 8 mg of hesperetin, respectively (aglucon equivalents). The sautéed onions (185 g), which provided 115 mg of quercetin, were fed in a three-egg omelet. The subjects consumed each flavonoid source along with carmine red (170 mg/capsule, NuCara Pharmacy, Waterloo, IA) within 10 min for breakfast on the morning of the study. Other breakfast items not containing flavonoids were provided (e.g., toast, bagels, cereal, milk, eggs, and cheese). All flavonoid sources and food items were purchased from local grocery stores. Baseline urine, blood, and fecal samples were collected before each flavonoid feeding. All urine was collected for 24 h into plastic containers in three fractions, 0−6, 6−12, and 12−24 h. Blood samples (10 mL) were collected into vacuum tubes containing EDTA at 2, 4, 6, 12, and 24 h after consumption of the isoflavone and naringenin test meals. Blood samples were collected 1, 3, 5, 12, and 24 h after the quercetin feeding. All feces were collected until carmine red was excreted. Urine and fecal samples were frozen at −20 °C until analysis. Blood samples were centrifuged at 3000*g*, 25 min, and 4 °C within 1 h of collection. The plasma was separated and stored at −80 °C until analysis. The subjects remained at the study site until the collection of the 6 h blood sample, after which they were allowed to leave and return for collection of 12 and 24 h blood samples.

Analytical Methods. *Flavonoid Analysis in Foods*. Isoflavones in soy milk were hydrolyzed and extracted according to the method of Murphy et al. (*19*). One gram of freeze-dried and ground grapefruit juice was extracted in 12 mL of 1 M HCl in a 125 mL screw-top Erlenmeyer flask for 2 h at 98 °C with stirring. The solution was cooled, and 18 mL of acetonitrile was added to the solution and stirred for 5 min. One milliliter of this solution was filtered with a 0.45 μm PTFE filter (Alltech Associates, Deerfield, IL) into an HPLC vial. Red onions were chopped and slightly sautéed, before being cooked in a three-egg omelet. Portions of the omelet were freeze-dried, homogenized, and extracted according to the method described above for grapefruit juice. Total aglycons of each flavonoid were obtained by HPLC analysis (below).

Urine Analysis. Urine samples were pooled in 0−6, 6−12, and 12−24 h increments for each subject. Five milliliter samples were incubated with 5 mL of 0.2 M sodium acetate buffer at pH 5.5 and 50 μL of β-glucuronidase/sulfatase (H_2 type, Sigma Chemical Co., St. Louis, MO) for 18 h in a 37 °C shaking water bath. Ten milliliters of 10 mM sodium phosphate buffer at pH 7.0 was added to the incubation solution and mixed well. The solution was applied to a 20 mL Extrelut QE column (EM Sciences, Gibbstown, NJ), and the column was eluted twice with 18 mL of ethyl acetate. The ethyl acetate collections were collected, pooled, and dried on a rotary evaporator at 25 °C. The residue was dissolved in 9.8 mL of 20% ethanol in water and 200 μL of 1 N HCl. Five milliliters of this mixture was loaded onto a preactivated SepPak C_{18} cartridge and washed twice with 2 mL of Milli-Q water. The flavonoids were eluted with 2 mL of 80% methanol in water and filtered through a 0.45 μm PTFE filter into an HPLC vial.

Plasma Analysis. One milliliter of plasma was incubated with 1 mL of 0.2 M sodium acetate at pH 5.5 and 50 μL of glucuronidase/sulfatase H2 type for 20 h at 37 °C on a shaking water bath. One milliliter of 10 mM sodium phosphate buffer (pH 7.0) was added to the incubation solution and mixed. The solution was added to a 5 mL Extrelut SE column (EM Sciences) and the flavonoids eluted twice with 2 mL of ethyl acetate into a 20 mL test tube. The solvent was evaporated under N_2, and the dried residue was dissolved in 200 μL of 80% methanol and then filtered with a 0.45 μm PTFE filter into an HPLC vial.

HPLC Analysis. A Beckman Coulter (Fullerton, CA) HPLC system was used for HPLC analysis consisting of a System Gold 126 solvent module, a System Gold 508 autosampler, and a System Gold 168 photodiode array detector. Forty microliter samples were injected onto a reversed-phase, 5 μm, C18 AM 303 column (250 × 4.6 mm) (YMC Co. Ltd., Wilmington, NC). The mobile phase consisted of 0.1% glacial acetic acid in water (A) and 0.1% glacial acetic acid in acetonitrile (B). Solvent B was increased from 30 to 50% over 10 min, increased to 100% over 7 min, and held for 1 min. The gradient was recycled to 30% over 2 min, flow rate = 1 mL/min. The wavelengths used for the preparation of standard curves, detection, and quantification of flavonoid peaks were 254 nm for isoflavones, flavonols, and flavones and 292 nm for flavanones, with LOD of 10 nmol/L for all compounds. Integration of peak area responses and evaluation of ultraviolet spectra were carried out using 32 Karat software (Beckman Coulter Inc.).

Data Analysis. The ratio of peak area of a flavonoid to THB (0.1 μmol/L) versus the flavonoid concentration was used as a standard calibration curve to estimate the concentration of flavonoids in the in vitro fecal fermentations and in foods. The rate of disappearance of flavonoids in fecal fermentation mixtures was estimated by plotting ln(% remaining flavonoid) versus time. The negative slope of this line was the apparent first-order flavonoid disappearance rate constant. Flavonoid disappearance phenotypes were identified using cluster analysis (*20*). Gut transit time (GTT) was determined by the time after ingestion for carmine red to appear in feces. GTT estimates were averaged for the three flavonoid feedings. The amounts of flavonoids in urine and plasma were calculated directly from external standard curves using flavonoids added to urine or plasma (n = 3 samples) and extracted in the same manner as the experimental samples with standard curves obtained by plotting peak area versus concentration. Urinary and plasma recoveries, respectively, were as follows: genistein, 84.0 ± 6.8 and 77.7 ± 5.2%; daidzein, 84.3 ± 5.7 and 78.6 ± 4.7%; naringenin, 89.1 ± 3.9 and 84.2 ± 4.1%; hesperetin, 88.9 ± 5.3 and 81.6 ± 3.2%; quercetin, 69.7 ± 10.5 and 60.2 ± 8.8%. Pharmacokinetic parameters were determined using a one-compartment linear model. Bioavailability was calculated as total urinary flavonoid excretion over 24 h, as a percentage of ingested dose. Plasma area under curve ($AUC_{0\rightarrow 24h}$) was calculated using the linear trapezoidal rule (*21*). The peak plasma concentration (C_{max}) and the time to reach it (t_{max}) were taken directly from the data. The elimination half-life ($t_{1/2}$) was calculated as $t_{1/2} = \ln 2/k$. Statistical evaluation of all experimental results was performed using the SAS system (version 8.1, SAS Institute, Cary, NC). Differences in flavonoid bioavailability, normalized AUCs, overall and individual degradation rates of flavonoids, and daidzein degradation phenotypes were estimated using one-way ANOVA, general linear models. All analyses were performed in duplicate, and all data are reported as mean ± SD. The statistical significance of all analyses was set at $\alpha = 0.05$.

RESULTS

In Vitro Flavonoid Degradation. Fecal disappearance rate differences were analyzed for genistein, apigenin, naringenin, kaempferol, luteolin, quercetin, myricetin, hesperetin, chrysin, flavone, daidzein, glycitein, 5,4′-dihydroxyflavone, 6,4′-dihydroxyflavone, 7,4′-dihydroxyflavone, and 5,3′-dihydroxyflavone (**Table 2**). The 5,7,4′-trihydroxyl flavonoids (genistein, naringenin, apigenin, kaempferol, quercetin, and luteolin) rapidly disappeared from the fecal incubations, $k \sim 0.46 \pm 0.10\ h^{-1}$ ($p < 0.0001$), except for the 5,7,4′-trihydroxyl flavonoid myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone), with $k = 0.04 \pm 0.03\ h^{-1}$. The methylated flavonoids, hesperetin and glycitein, rapidly disappeared with $k = 0.24 \pm 0.21$ and $0.18 \pm 0.09\ h^{-1}$, respectively. All other flavonoids

(chrysin, flavone, daidzein, 5,4′-dihydroxyflavone, 6,4′-dihydroxyflavone, 7,4′-dihydroxyflavone, and 5,3′-dihydroxyflavone) were slowly degraded, $k \sim 0.05 \pm 0.03$ h^{-1}.

Isoflavone Bioavailability. After ingestion of soy milk, genistein and daidzein plasma concentrations peaked ~5 h after dosing, with $t_{1/2}$ of 2.1 and 1.1 h, respectively (**Table 3**). Peak concentrations were 0.7 ± 0.3 μmol/L for genistein and 1.0 ± 0.4 μmol/L for daidzein. Mean apparent absorptions of genistein and daidzein (flavonoid excreted in urine as a percentage of ingested dose) were 7.2 ± 4.8 and 42.6 ± 16.0%, respectively (**Table 3**). In vitro fecal genistein disappearance rates of the 10 subjects clustered into three significantly different groups ($p < 0.0001$): high ($k = 1.28 \pm 0.45$ h^{-1}, $n = 3$), moderate ($k = 0.35 \pm 0.01$ h^{-1}, $n = 3$), and low ($k = 0.11 \pm 0.07$ h^{-1}, $n = 4$, **Figure 2A**). Urinary genistein excretion in low genistein degraders was 11.5 ± 4.9% of ingested dose, significantly greater than urinary genistein excretion in moderate (3.5 ± 1.6%) and high genistein degraders (4.9 ± 1.2%, $p < 0.05$, **Figure 2B**). There was no difference in plasma AUC of genistein across individuals of high, moderate, and low genistein disappearance rates (high, 0.059 ± 0.14 μmol·h·L^{-1}/μmol ingested; moderate, 0.086 ± 0.061 μmol·h·L^{-1}/μmol ingested; and low, 0.114 ± 0.044 μmol·h·L^{-1}/μmol ingested, $p > 0.1$), nor did plasma genistein AUC differ significantly from plasma AUC of daidzein per micromole ingested (**Table 3**).

Flavanone Bioavailability. After ingestion of grapefruit juice, naringenin and hesperetin plasma concentrations peaked at 5.1 and 12 h, respectively, with peak plasma concentrations of 0.3 ± 0.2 and 0.05 ± 0.09 μmol/L, respectively (**Table 3**). Plasma half-lives were 5.7 h for naringenin and 3.6 h for hesperetin. The mean urinary excretion as percentage of ingested dose of naringenin was 3.2 ± 1.7%, and that for hesperetin in males was 7.3 ± 3.2%. Hesperitin was not recovered in females (**Table 3**). In vitro fecal naringenin disappearance rates clustered into three significant groups: high (mean $k = 0.63 \pm 0.20$ h^{-1}, $n = 2$), moderate (mean $k = 0.20 \pm 0.01$ h^{-1}, $n = 3$), and low (mean $k = 0.05 \pm 0.03$ h^{-1}, $n = 4$). There was no difference in urinary naringenin excretion or plasma AUC across these three phenotypic groups ($p > 0.05$). The in vitro fecal hesperetin disappearance rates did not cluster into significantly different subgroups (data not shown).

Flavonol Bioavailability. After the ingestion of cooked red onion, plasma quercetin concentrations peaked at 1.5 h with a half-life of ~9 h. Mean peak plasma concentration was 0.8 ± 0.6 μmol/L, AUC of 6.1 ± 6.1 μmol·h/L (not corrected for μmol intake). The pharmacokinetics of quercetin was different from that of the other flavonoids, in that the mean time of peak plasma concentration was 1.5 ± 1.3 h, and significantly earlier than the other flavonoids with a mean t_{max} value of 4.4 ± 1.2 h ($p = 0.00064$). The mean elimination half-life was 9 h and ranged from 1 to 20 h compared to the half-lives of the other flavonoids, which ranged from 1 to 6 h. Urinary excretion of quercetin was 5.6% of ingested dose (**Table 3**). In vitro fecal quercetin disappearance did not sort into distinct clusters (data not shown).

Overall Flavonoid Bioavailability Comparison. When flavonoids were compared per micromole ingested, plasma AUC values were not significantly different for genistein and daidzein (**Table 3**). The mean plasma AUC values for genistein and daidzein were significantly greater than the AUC values for naringenin, quercetin, and hesperetin ($p = 0.001$). Naringenin had the least plasma AUC and apparent absorption, both significantly less than all of the other flavonoids tested ($p < 0.0001$, **Table 3**). Apparent absorptions (urinary excretion) were not significantly different between quercetin, hesperetin, and genistein (6.6%, $p > 0.05$). The apparent absorption of daidzein was significantly greater than that of the other flavonoids ($p < 0.0001$).

Table 2. In Vitro Anaerobic Human Fecal Flavonoid Degradation Rates ($n = 33$ Subjects)

flavonoid	in vitro degradation rate (h^{-1})	flavonoid	in vitro degradation rate (h^{-1})
hesperetin	0.75 ± 0.05a[a]	chrysin	0.08 ± 0.05c
naringenin	0.47 ± 0.28b	5,4′-dihydroxyflavone	0.08 ± 0.05c
genistein	0.38 ± 0.32b	5,3′-dihydroxyflavone	0.07 ± 0.04c
apigenin	0.37 ± 0.18b	flavone	0.07 ± 0.02c
quercetin	0.35 ± 0.31b	daidzein	0.07 ± 0.03c
luteolin	0.21 ± 0.16b	7,4′-dihydroxyflavone	0.05 ± 0.02c
glycitein	0.18 ± 0.09bc	6,4′-dihydroxyflavone	0.04 ± 0.03c
kaempferol	0.12 ± 0.17b	myricetin	0.04 ± 0.04c

[a] Letters indicate significant differences, $p < 0.05$.

DISCUSSION

The flavonoids that were seemingly more rapidly degraded in in vitro fecal incubations from 33 subjects included the isoflavone genistein, the flavones apigenin and luteolin, the flavonols quercetin and kaempferol, and the flavanone naringenin. All of these flavonoids have hydroxyl groups on the 5-, 7-, and 4′-positions of the flavonoid backbone structure (**Figure 1**). These results confirmed previous results which showed that 5,7,4′-trihydroxyl flavonoids were most rapidly degraded by fecal microbes from 11 human subjects compared with flavonoids not having these structural features (*14*). However, myricetin, which also has hydroxyl groups in the 5-, 7-, and 4′-positions, was not degraded rapidly. The rapidly degraded 5,7,4′-trihydroxyl flavonoids in this study possessed three to five hydroxyl groups and were similar in their hydrophobicity when analyzed by HPLC (data not shown). Myricetin has six hydroxyl groups on the flavonoid backbone structure and is much more hydrophilic compared with other 5,7,4′-trihydroxyl flavonoids, which may hinder its bacterial degradation. Other flavonoids that were rapidly degraded were the methoxylated isoflavone and flavanone, glycitein and hesperetin, respectively.

Table 3. Bioavailability Parameters of Ingested Flavonoids in Human Subjects[a]

isoflavone	N	ingested dose (μmol)	in vitro fecal disappearance rate[b] (h^{-1})	relative plasma AUC (μmol·h/L/ μmol ingested)	C_{max} (μmol/L)	t_{max} (h)	$t_{1/2}$ (h)	amount excreted over 24 h (μmol)	apparent absorption (urinary recovery) (% of ingested dose)
genistein	10	103.6	0.55 ± 0.56a	0.088 ± 0.046a	0.7 ± 0.3	4.8 ± 3.0a	2.1 ± 1.6	7.9 ± 5.3	7.2 ± 4.8b
daidzein	10	61.8	0.05 ± 0.03b	0.210 ± 0.060a	1.0 ± 0.4	5.3 ± 0.9 a	1.1 ± 0.8	26.8 ± 10.1	42.6 ± 16.0a
naringenin	9	1549.5	0.23 ± 0.25a	0.002 ± 0.001c	0.3 ± 0.2	5.1 ± 1.6 a	5.7 ± 5.9	50.0 ± 27.7	3.2 ± 1.7c
hesperetin[c]	5	25.5	0.75 ± 0.05a	0.027 ± 0.035b	0.05 ± 0.09	12.0 ± 5.5 a	3.6 ± 4.9	0.9 ± 0.4	7.3 ± 3.2b
quercetin	8	380.5	0.35 ± 0.31a	0.016 ± 0.016b	0.8 ± 0.6	1.5 ± 1.3 b	9.1 ± 8.9	21.1 ± 4.4	5.6 ± 3.7b

[a] Bioavailability parameter values are means ± standard deviations.Means in a column without a common letter are significantly different, $p < 0.05$. [b] Disappearance rates measured prior to the feeding studies. [c] Hesperetin was not recovered in female subjects.

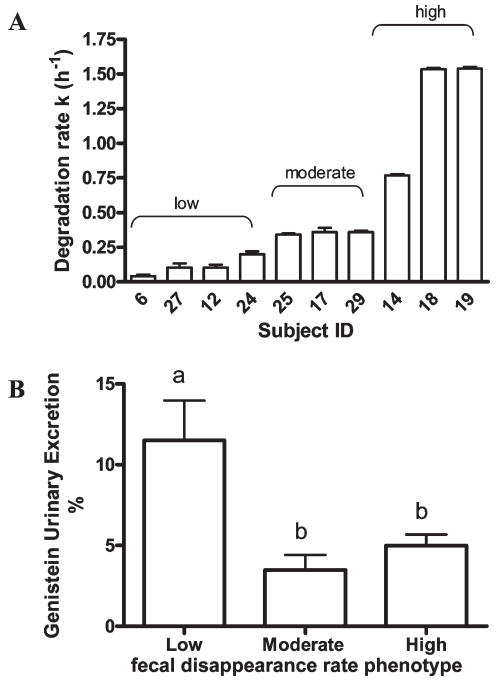


Figure 2. In vitro fecal disappearance phenotypes for genistein and correlation with urinary excretion: (**A**) cluster analysis of genistein in vitro fecal disappearance rates; (**B**) amount of genistein excreted in urine after 24 h in subjects with high, moderate, and low in vitro fecal genistein disappearance phenotypes (bars with different letters were significantly different, $p < 0.05$).

These methoxylated flavonoids were rapidly demethylated in vitro before further microbial degradation, and their calculated degradation rates were based on the demethylation reaction, and not on disappearance of the demethylation product. In an in vitro human fecal incubation mixture, hesperetin was rapidly demethylated to eriodictyol, which has a 5,7,4′-trihydroxyl flavonoid structure. Eriodictyol then rapidly disappeared, which supported our findings that 5,7,4′-trihydroxyl flavonoids are rapidly degraded (data not shown). Hesperetin was demethylated in an in vitro pig cecum model to eriodictyol and then further degraded to 3-(3-hydroxyphenyl)propionic acid and phloroglucinol (*22*). We showed that the major degradation pathway of glycitein is demethylation to 6,7,4′-trihydroxyl isoflavone (*23*). Hesperetin disappeared significantly more rapdily than did glycitein (**Table 2**), perhaps as a result of a more rapid demethylation reaction from the 4′-position in hesperetin compared with the 6-position in glycitein. The methoxylated isoflavones formononetin, biochanin A, and glycitein were demethylated regardless of the position of the methoxyl group, but demethylation rates were not reported (*24*). Perhaps the 5-hydroxyl of hesperetin was responsible for this faster reaction; hesperetin has a 5-hydroxyl group, and glycitein does not.

Because 5,7,4′-trihydroxyl flavonoids disappeared from fecal incubations rapidly in vitro, we tested the hypothesis that the compounds genistein, naringenin, quercetin, and hesperetin would not be very absorbable in humans because these flavonoids would be more likely to be degraded by gut microbes before they could be absorbed, whereas flavonoids such as daidzein lacking one of the 5-, 7-, and 4′-hydroxyl groups would be more absorbable because they disappear at a slower rate and have more time to be absorbed compared with 5,7,4′-trihydroxyl flavonoids.

The apparent absorption of daidzein as reflected in urinary excretion was significantly greater than that for genistein, hesperitin, naringenin, and quercetin (**Table 3**). The difference in absorption between daidzein and genistein agrees with other studies of subjects ingesting similar doses of genistein and daidzein in soy foods (see, e.g., ref 25). Doses of ~97 μmol of daidzein and 71 μmol of genistein resulted in an average daidzein bioavailability of 19.8%, significantly greater than the mean genistein bioavailability of 5.3% (*25*).

Apparent absorption of naringenin reflected in urinary excretion was ~3% and significantly less than that of the other flavonoids, hesperetin, genistein, and quercetin, that were rapidly degraded in vitro in fecal samples (**Table 2**). The plasma AUC of naringenin was also less than that of the other rapidly degraded flavonoids (**Table 3**). Low naringenin bioavailability was found in previous studies reporting 4−5% apparent absorption (urinary excretion) from a single oral dose of 1837 μmol of pure naringin ingested by human subjects (*26*). However, a wide range of 5−57% naringenin bioavailability (as urinary excretion) was reported in six subjects ingesting 26 μmol of naringin/kg of body weight (*27*). Because our study was the first to compare the apparent absorption of these flavonoids, perhaps the far greater dose of naringenin than of the other flavonoids may have limited naringenin uptake. The small number of subjects in our study may have prevented an ability to distinguish effects of interindividual variability in putative gut microbial degradation of naringenin, as seen in degradation rate clusters (see Results, above), on the uptake of this compound.

Most of the available research on hesperetin bioavailability has previously been determined from ingestion of orange juice, because hesperetin is the major flavanone in orange juice, whereas naringenin predominates in grapefruit juice. Hesperetin bioavailability ranged from 3 to 6% (*26*, *27*), which roughly agrees with the mean hesperetin bioavailability of 7% (**Table 3**). We observed a sex difference in hesperetin bioavailability in that hesperetin was not recovered in the urine or plasma of females, so hesperetin bioavailability was based on the five males (**Table 3**). The reason for the apparent lack of hesperitin absorption in women is unknown. Perhaps there was sex-specific conversion of hesperitin to a metabolite that was not detectable by our HPLC analyses.

Apparent bioavailability of quercetin was ~6% of ingested dose. These data supported previous data of 6% quercetin bioavailability, $t_{max} = 0.68$ h and $t_{1/2} = 10.9$ h, after 12 human subjects ingested 331 μmol of quercetin in onions (*28*), a dose comparable with our study (381 μmol). Lesser apparent quercetin absorption of 1% was shown after intake of 300 g of lightly fried yellow onions in five subjects (*29*). The reason for the difference in these bioavailability values is not clear, but may be due to substances in the omelet or in red onions that facilitated the absorption of quercetin.

Plasma AUCs of the flavonoids generally corresponded with urinary excretion, except in the case of genistein. Although plasma AUC of daidzein was >2-fold more than that of genistein when expressed per μmicromoles ingested, there was no significant difference in the AUC of genistein and daidzein, perhaps because of great interindividual variability and small numbers of subjects. Because 4 of 10 subjects had low fecal degradation rates for genistein (**Figure 2**), mean plasma AUC for genistein may be expected to be more similar to that for daidzein, on the basis of a previous study in which plasma daidzein was either greater than or similar to plasma genistein depending on the subset of subjects studied (*10*). Two women who had ~10-fold greater apparent absorption

of both isoflavones absorbed both isoflavones to a similar extent, whereas five other women who had lesser overall isoflavone absorption showed greater absorption of daidzein than of genistein (*10*).

We observed significant interindividual variation within compounds in fecal flavonoid degradation rates in vitro (**Table 2**). For all of the flavonoids except hesperetin and quercetin, cluster analysis revealed three significant groupings ($p < 0.0001$, data not shown); high, moderate, and low flavonoid disappearance rates. Low flavonoid degraders may experience greater bioavailability of flavonoids compared with high flavonoid degraders because, in the low flavonoid degraders, the flavonoids have more time to be absorbed before they disappear. For example, women ($n = 12$) with low fecal isoflavone disappearance rates experienced greater apparent absorption of isoflavones over 24 h compared with women with high fecal isoflavone disappearance rates ($n = 13$) (*12*). Although previous studies indicate that interindividual variability in fecal degradation rates is an important factor in interpreting flavonoid bioavailability data (*10*−*12*) (**Table 2**), the current study showed differences across these fecal degradation rate clusters only for the apparent absorption (urinary excretion) of genistein (**Figure 2**). A 2-fold greater difference in fecal degradation rate corresponded with ∼2-fold less absorption, and greater fecal degradation rate did not further decrease genistein uptake. Although similar differences across fecal degradation rate clusters occurred for naringenin compared with genistein, perhaps the high dose of naringenin prevented the observation of an effect of these fecal degradation rate clusters on naringenin uptake.

These results provide a partial explanation as to why flavonoids with minor differences in their chemical structure may exert different biological effects. Differences in flavonoid chemical structures affect their apparent intestinal microbial degradation and human intestinal uptake, which is a key factor in the biological activity of flavonoids. A recent hamster study supported the importance of relative isoflavone absorption in determining the efficacy of a soy protein diet to lower cholesterol. Only hamsters clustering as high absorbers as reflected in their urinary excretion of isoflavones exhibited significant reduction of total and non-HDL cholesterol compared with casein-fed controls; these high absorbers accounted for the observed lowering of cholesterol by the soy protein diet (*30*). More data are needed on the relationship of flavonoid chemical structure to biological effects across and within flavonoid subgroups, but, clearly, intestinal microbial metabolism of these compounds must be considered.

LITERATURE CITED

(1) Harborne, J. B. Nature distribution and function of plant flavonoids. *Prog. Clin. Biol. Res.* **1986**, *280*, 17–27.

(2) Hertog, M. G. L.; Hollman, P. C.H.; van de Putte, B. Content of potentially anticarcinogenic flavonoids in tea infusions, wine and fruit juices. *J. Agric. Food Chem.* **1993**, *41*, 1242–1246.

(3) Cook, N. C.; Samman, S. Flavonoids − chemistry, metabolism, cardioprotective effects, and dietary sources. *Nutr. Biochem.* **1996**, *7*, 66–76.

(4) Hooper, L.; Kroon, P. A.; Rimm, E. B.; Cohn, J. S.; Harvey, I.; Le Cornu, K. A.; Ryder, J. J.; Hall, W. L.; Cassidy, A. Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. *Am. J. Clin. Nutr.* **2008**, *88*, 38–50.

(5) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S.

(6) Day, A. J.; Gee, J. M.; DuPont, S.; Johnson, I. T.; Williamson, G. Absorption of quercetin-3-glucoside and quercetin-4′′-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent transporter. *Biochem. Pharmacol.* **1999**, *65*, 1199–1206.

(7) Crespy, V.; Morand, C.; Manach, C.; Besson, C.; Demigne, C.; Remesy, C. Part of quercetin absorbed in the small intestine is conjugated and further secreted in the intestinal lumen. *Am. J. Physiol.* **1999**, *277*, 120–126.

(8) Liu, Y.; Hu, M. Absorption and metabolism of flavonoids in the Caco-2 cell culture model and a perused rat intestinal model. *Drug Metab. Dispos.* **2002**, *30*, 370–377.

(9) Turner, N. J.; Thomson, B. M.; Shaw, I. C. Bioactive isoflavones in functional foods: the importance of gut microflora on bioavailability. *Nutr. Rev.* **2003**, *61*, 204–213.

(10) Xu, X.; Harris, K. S.; Wang, H. J.; Murphy, P. A.; Hendrich, S. Bioavailability of soybean isoflavones depends upon gut microflora in women. *J. Nutr.* **1995**, *125*, 2307–2315.

(11) Zheng, Y.; Hu, J.; Murphy, P. A.; Alekel, D. L.; Franke, W. D.; Hendrich, S. Rapid gut transit time and slow fecal isoflavone disappearance phenotype are associated with greater genistein bioavailability in women. *J. Nutr.* **2003**, *133*, 3110–3116.

(12) Zheng, Y.; Lee, S. O.; Verbruggen, M. A.; Murphy, P. A.; Hendrich, S. The apparent absorptions of isoflavone glucosides and aglucons are similar in women and are increased by rapid gut transit time and low fecal isoflavone degradation. *J. Nutr.* **2004**, *134*, 2534–2539.

(13) Zhang, Y.; Wang, G. J.; Song, T. T.; Murphy, P. A.; Hendrich, S. Urinary disposition of the soybean isoflavones daidzein, genistein and glycitein differs among humans with moderate fecal isoflavone degradation activity. *J. Nutr.* **1999**, *129*, 957−962; erratum *J. Nutr.* **2001**, *131*, 147−148.

(14) Simons, A. L.; Renouf, M.; Hendrich, S.; Murphy, P. A. Human gut microbial degradation of flavonoids: structure−function relationships. *J. Agric. Food Chem.* **2005**, *53*, 4258–4263.

(15) Loke, W. M.; Jenner, A. M.; Proudfoot, J. M.; McKinley, A. J.; Hodgson, J. M.; Halliwell, B.; Croft, K. D. A metabolite profiling approach to identify biomarkers of flavonoid intake in humans. *J. Nutr.* **2009**, doi: 10.3945/jn.109.113613.

(16) USDA. Flavonoid Content of Selected Foods − 2003, http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.html.

(17) Song, T. T.; Barua, K.; Buseman, G.; Murphy, P. A. Soy isoflavone analysis: quality control and new internal standard. *Am. J. Clin. Nutr.* **1998**, *68*, 1474S–1479S.

(18) Chang, Y. C.; Nair, M. G.; Santell, R. C.; Helferich, W. G. Microwave-mediated synthesis of anticarcinogenic isoflavones from soybeans. *J. Agric. Food Chem.* **1994**, *42*, 1869–1871.

(19) Murphy, P. A.; Song, T.; Buseman, G.; Barua, K.; Beecher, G. R.; Trainer, D.; Holden, J. Isoflavones in retail and institutional soy foods. *J. Agric. Food Chem.* **1999**, *47*, 2697–2704.

(20) Johnson, R. A.; Wichern, D. W. *Applied Multivariate Statistical Analysis*, 5th ed.; Prentice-Hall: Upper Saddle River, NJ, 2002; pp 675−694.

(21) Shen, D. D. Toxicokinetics. In *Cassarett and Doull's Toxicology: The Basic Science of Poisons*, 7th ed.; Klassen, C. D., Ed.; McGraw-Hill: New York, 2008; p 310.

(22) Labib, S.; Erb, A.; Kraus, M.; Wickert, T.; Richling, E. The pig caecum model: a suitable tool to study the intestinal metabolism of flavonoids. *Mol. Nutr. Food Res.* **2004**, *48*, 326–332.

(23) Simons, A. L.; Renouf, M.; Hendrich, S.; Murphy, P. A. Metabolism of glycitein (7,4′-dihydroxy-6-methoxy-isoflavone) by human gut microflora. *J. Agric. Food Chem.* **2005**, *53*, 8519–8525.

(24) Hur, H.; Rafii, F. Biotransformation of the isoflavonoids biochanin A, formononetin and glycitein by *Eubacterium limosum*. *FEMS Microbial. Lett* **2000**, *192*, 21–25.

(25) Xu, X.; Wang, H. J.; Murphy, P. A.; Cook, L.; Hendrich, S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J. Nutr.* **1994**, *124*, 825–832.

(26) Ishii, K.; Furuta, T.; Kasuya, Y. Mass spectrometric identification and high-performance liquid chromatographic determination of

a flavonoid glycoside naringin in human urine. *J. Agric. Food Chem.* **2000**, *48*, 56–59.
(27) Manach, C.; Morand, C.; Gil-Izquierdo, A.; Bouteloup-Demange, C.; Remesy, C. Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *Eur. J. Clin. Nutr.* **2003**, *57*, 235–242.
(28) Graefe, E. U.; Wittig, J.; Mueller, S.; Riethling, A. K.; Uehleke, B.; Drewelow, B.; Pforte, H.; Jacobasch, G.; Derendorf, H.; Veit, M. Pharmacokinetics and bioavailability of quercetin glycosides in humans. *J. Clin. Pharmacol.* **2001**, *41*, 492–499.
(29) Aziz, A. A.; Edwards, C. A.; Lean, M. E. J.; Crozier, A. Absorption and excretion of conjugated flavonols including quercetin-4′-*O*-*β*-glucoside and isorhamnetin-4′-*O*-*β*-glucoside by human volunteers after the consumption of onions. *Free Radical Res.* **1998**, *29*, 257–269.
(30) Zhong, Y.; Renouf, M.; Lee, S.-O.; Hauck, C. C.; Murphy, P. A.; Hendrich, S. High urinary isoflavone excretion phenotype decreases plasma cholesterol in Golden Syrian hamsters fed soy protein. *J. Nutr.* **2006**, *136*, 2773–2778.

Received for review July 2, 2009. Revised manuscript received October 16, 2009. Accepted October 23, 2009. This work was partly funded by the Center for Designing Foods to Improve Nutrition, Iowa State University, U.S. Department of Agriculture Special Grant 2003-0679. This work was presented in part at Experimental Biology, April 2−6, 2005, San Diego, CA (Nutrition and Metabolism Program, Dietary bioactive components I: Gastrointestinal and postabsorptive metabolism, abstract based oral session 5060-ASNS).